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Award Number: DAMD17-99-1-9142

TITLE: Mechanism of Growth Factor Attenuation of Cell Death in Chemotherapy Treated Breast Cancer Cells

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REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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DTIC QUALITY INSPECTED 3
20010108 169

REPORT DOCUMENTATION PAGE

OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual Summary (1 Aug 99 - 31 Jul 00)

4. TITLE AND SUBTITLE
Mechanism of Growth Factor Attenuation of Cell Death in Chemotherapy Treated Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-99-1-9142

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Upregulation of IGF-I (Insulin-like Growth Factor-I) receptor dependent pathways like phosphatidylinositol 3-kinase (PI3K) may diminish chemosensitivity of breast cancer cells via c-Jun N-terminal kinase (JNK) response. In this project we set out to determine if IGF-I activates the PI3K survival pathway. We also predicted that IGF-I treatment of breast cancer cells would inhibit the stress-induced pathway JNK which is activated by some chemotherapy drugs.

Initially, we performed PI3K assays and determined that IGF-I treatment of MCF-7 breast cancer cells strongly activated PI3K. Similarly, IGF-I treatment activated both downstream kinases Akt and p70S6.

Next, we assessed if JNK activity was induced by stress treatment of MCF-7 cells, using doxorubicin, taxol, taxotere, and ultraviolet irradiation (UV). Taxol, taxotere and UV were the strongest activators of JNK activity and were used for subsequent IGF-I co-treatment assays. Surprisingly, IGF-I treatment alone lead to significant activation of JNK. Further, inhibition of PI3K inhibited IGF-I induction of JNK, suggesting that PI 3-kinase may convey survival responses through JNK. When cells were co-treated with IGF-I and stress treatment, JNK activity was additive compared to either treatment alone. Transfection experiments support that IGF-I mediated survival responses are conveyed through PI3K and Akt.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	44
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

Cancer patients experience several toxicities from chemotherapy. One frequent toxicity is peripheral neuropathy, and strategies to relieve chemotherapy-induced neuropathy have had little success. Clinical trials using IGF-I have been proposed to combat chemotherapy-related neurotoxicities. IGF-I has trophic effects on motor neurons and is used for treatment of amyotrophic lateral sclerosis (ALS or Lou Gerig's disease) and diabetic neuropathy. However, there is concern about the potential for IGF-I to stimulate tumor growth or inhibit chemotherapy effects in patients receiving various treatments.

Body

Research accomplishments: We are studying the effects of IGF-I on breast cancer cells, focusing particularly on its ability to inhibit chemotherapy activity. We hypothesized that IGF-I acts through its receptor to induce at least one cell survival pathway. Aim 1 of this project was to characterize IGF-I regulation of the PI3K-survival related survival pathway in breast cancer cells, in effort to identify anti-apoptotic pathways. Our studies show that IGF-I treatment of MCF-7 breast cancer cells rapidly elevates the activity of PI3K, and this activity is not altered by treatment with doxorubicin. We also confirmed the ability of IGF-I to activate kinases downstream of PI3K, like Akt and p70S6 kinase. Activation of PI3K and Akt was inhibited by co-treatment with PI3K inhibitors, wortmannin and LY294002; and IGF-I activation of p70S6 kinase activity was inhibited using co-treatment with rapamycin. Since the stability of each of the kinase inhibitors is short, we had concern that testing the role of each kinase by co-treatment of cells with kinase inhibitors after approximately four days of culture may lead to inconclusive results which we initially proposed. Therefore, cells were transfected with wildtype and mutant Akt, the PI3K dependent kinase most likely to convey IGF-I survival responses, to better delineate the role of PI3K and Akt in IGF-I survival responses. Overexpression of wildtype Akt inhibited chemotherapy induced cell death, while overexpression of mutant Akt augmented chemotherapy mediated cell death (see attached manuscript). These data support the important roles of PI3K and Akt in survival of breast cancer cells. Upregulation of this signal transduction pathway may account for one mechanism for chemoresistance in breast cancer cells.

Aim 2 of this project was to determine IGF-I and chemotherapy drug effects on the intracellular JNK stress response and to characterize potential interactions between the PI3K survival and the JNK stress-induced signaling with combined exposure to IGF-I and chemotherapy drugs. Our results indicate that JNK mediated cell suicide is enhanced by Taxol and Taxotere treatment, however, doxorubicin treatment does not alter JNK activity. Surprisingly, IGF-I treatment of cells markedly enhanced JNK activity as well. Co-culture with a PI3K inhibitor reduced IGF-I mediated JNK activity. In addition, when cells were co-treated with IGF-I and chemotherapy, JNK response was enhanced by co-treatment, rather than abrogated by IGF-I as we had initially predicted. In order to better delineate the function of JNK in our model, MCF-7 cells were transfected with wildtype and dominant negative forms of JNK. Stable transfectants were cultured in Taxol alone, IGF-I alone, or Taxol + IGF-I. Our results support a role for JNK in mediating cell death. Our data do not support a role for JNK3 in mediating IGF-I survival responses, but we may not have observed this interaction for various reasons (discussed in submitted manuscript).

Training accomplishments: Dr. Horwitz's laboratory has been instrumental in the development of our various *in vitro* kinase assays in my laboratory. For example, Dr. Carol Lange advised me on optimization of the JNK assays for our model and interpretation of our results. She also collaborated closely with me in developing PI3K assays for use in both my laboratory and the Horwitz laboratory. Finally, Dr. Dave Bain, also in the Horwitz laboratory, advised me regarding the synthesis of an Akt specific kinase that could be used to immobilize Akt from cell lysates and perform *in vitro* kinase assays.

In addition to these accomplishments, a graduate student in my laboratory successfully identified a quantitative assay to measure apoptosis in MCF-7 cells. MCF-7 cells are notoriously difficult to identify morphologic changes associated with apoptosis.

Key Research Accomplishments:

- *In vitro* kinase and apoptosis assay development
- Enhanced activity of Akt results in resistance to chemotherapy-induced apoptosis
- IGF-I induction of JNK is PI3K-dependent
- IGF-I and stress treatments of breast cancer cells leads to increased JNK activity compared to either treatment alone
- Confirmation of JNK mediated apoptotic effects

Reportable Outcomes

Abstracts

Van Den Berg, C.L., Mamay C., Molina, M. Mechanisms of Insulin-like growth factor-I (IGF-I) chemoprotective effect in MCF-7 breast cancer cells. *Proc Am Assoc Cancer Res* 41:406, 2000.

Manuscripts

Mamay, C.L., Wolf, D.M., Molina, D.M., Van Den Berg, C.L. "The cooperative and opposing roles of JNK and Akt in IGF-I mediated survival of chemotherapy treated breast cancer cells". Submitted to *Cancer Research.*

Funding Applied for Based on Work Supported by this Award

Grants Pending:

"IGF-I survival effects on p53 induced apoptosis." National Cancer Institute, October 2000 to September 2003. Principal Investigator. Total Budget: $563,750

"p53 dependence of IGF-I survival responses through JNK and Akt." Department of Defense Cancer Research Program, June 1999 to June 2003.
Principal Investigator. Total Budget: $453,000

The cooperative and opposing functions of JNK and Akt in IGF-I mediated survival of chemotherapy treated breast cancer cells *

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Running Title: The functions of JNK and Akt

Keywords: IGF-I, breast cancer, JNK, Akt , apoptosis

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Abstract

Clinical use of insulin-like growth factor-I (IGF-I) has been proposed to combat chemotherapy-related neurotoxicities. However, IGF-I may stimulate tumor growth or inhibit chemotherapy effects in cancer patients receiving treatment. Since many breast tumors and cancer cell lines overexpress IGF-I receptor, we have tested IGF-I effects on chemotherapy treated breast cancer cells. IGF-I induces proliferation and protects from chemotherapy-induced apoptosis, suggesting IGF-I and chemotherapy treatment outcomes are modulated by signal transduction proteins. We demonstrate that chemotherapy treatment induces c-Jun N-terminal kinase (JNK), a kinase that conveys cellular stress and death signals. Most notable is our observation that IGF-I alone induces a more potent JNK response than chemotherapeutic agents; this activity is reversed by inhibition of phosphatidylinositol 3-kinase (PI 3-kinase). Co-treatment of cells with chemotherapy and IGF-I, to assess interactions of opposing stimuli on cellular fate, leads to an additive JNK response. These results indicate that IGF-I may confer survival effects via PI 3-kinase and either of its downstream effectors Akt or JNK. Stable transfections were performed to test the importance of Akt and JNK in IGF-I mediated survival. Overexpression of Wt Akt enhanced cell survival with Taxol treatment alone and in the presence of Taxol and IGF-I co-treatment. In contrast, overexpression of Wt JNK3 significantly enhanced Taxol induced apoptosis and inhibited IGF-I's survival effect. Interestingly, Akt constitutive activity was increased by Wt JNK3 overexpression compared to parental controls. Akt activity was also enhanced by either Taxol treatment alone or in combination with IGF-I in both Wt JNK3 transfectants and parental cells. These results suggest that PI 3-kinase is proximal to both JNK and Akt and that JNK may be an intermediate kinase that alters Akt function. Continued study

of the functions of JNK and its interactions with Akt will be required to delineate the complexity of opposing signals and cellular outcome.

Introduction

Activation of IGF-IR by its ligand imparts both survival and proliferative effects in various experimental models including neuronal and cancer cells. The importance of IGF-IR action in breast cancer has been clearly demonstrated. Its expression has been observed in 87% of breast cancer specimens (1) and overexpressed IGF-IR in human tumor specimens is functional, having increased kinase activity compared to normal mammary tissue (2). Furthermore, Cullen and colleagues (3, 4) have shown that breast cancer evolution is associated with the induction of IGF-II secretion, another IGF-IR ligand, from stromal cells neighboring malignant breast tissue. Investigators have also shown that systemically administered IGF-I stimulates tumor growth in mice in a dose-dependent fashion using NIH3T3 cells overexpressing IGF-IR (5). Inhibition of IGF-IR activity by overexpression of a dominant negative form of IGF-IR in MDA-MB-231 and MDA-MB-435 breast cancer cell lines inhibited the adhesion, invasion and metastasis of these cells, as well as enhanced their sensitivity to Taxol-induced cell death (6). Some of these IGF-IR mediated tumorigenic properties may be attributed to its central role in regulation of proteins important for either matrix attachment or cell survival.

IGF-IR activation leads to several downstream events. After IGF-IR autophosphorylation, protein complexes assemble with either IRS-1 or IRS-2 and activate second messengers such as MAPK and PI 3-kinase-dependent Akt and/or p70 S6 kinase. Ultimately, PI 3-kinase activation of Akt is thought to convey most IGF-I dependent survival effects (7). Induction of p70 S6 kinase may also be indirectly achieved by Akt in a PI 3-kinase dependent fashion to stimulate MCF-7 proliferation (8). Certainly, crosstalk and regulation among these cytoplasmic

messengers may lead to response of more than one signaling pathway and diverse biological endpoints. We hypothesized that IGF-IR chemoprotective effects in our model result from stimulation of PI 3-kinase and its downstream effector Akt or other PI 3-kinase sensitive proteins.

IGF-I treatment may also impose either direct effects on cell death or stress pathways or indirect effects by crosstalk between survival and death signaling pathways. To date, these interactions have primarily been reported to occur through downstream proteins like the Bcl-2 related protein, BAD (9) and caspase 9 (10). IGF-I may also confer cytoprotection by inhibiting upstream kinases in pathways associated with cell death. For example, investigators have shown that IGF-I may protect fibroblasts from UV-B induced apoptosis via JNK (11), while others have shown that pretreatment with IGF-I inhibits induction of JNK (12). Thus, IGF-I inhibition of JNK has been set forth as another mechanism for its cytoprotective properties in fibroblasts.

JNK was initially identified as an important component of a signaling pathway induced by various forms of cellular stress, including UV irradiation, cytokines, heat stress, and DNA damage. In turn, activated JNK phosphorylates transcription factors including c-Jun, ATF-2, Elk-1, and p53 that alter gene transcription via association with AP-1 or other protein complexes to regulate cellular response. Although other investigators have proposed that IGF-I survival effects may be mediated by inhibition of JNK signaling, much remains to be determined as to whether cancer cells overexpressing growth factor receptors may harbor abnormal regulatory mechanisms to cope with environmental stress and escape cell death.

Since many chemotherapy drugs induce JNK, possibly as a result of DNA damage or microtubule interference (13, 14), we first sought to determine if chemotherapy treatment of breast cancer cells would induce JNK activity in our model. Furthermore, we examined whether chemotherapy response through JNK might be altered by IGF-I. It has been previously shown that growth factors may increase JNK activity, however, little is known about the importance of JNK in transducing growth factor dependent effects. Since growth factor and stress stimuli result in diverse biological endpoints, we designed experiments to determine if IGF-I treatment of breast cancer cells would either abrogate or enhance drug induced JNK activity as a potential mechanism for its inhibition of cell death.

In this paper, we confirm that chemotherapy drugs frequently used for the treatment of breast cancer induce JNK activity in breast cancer cells. Interestingly, we show that treatment with IGF-I alone potently activates JNK. In MCF-7 breast cancer cells this action is significantly reduced by PI 3-kinase inhibition. Co-treatment of breast cancer cells with both chemotherapy and IGF-I results in an additive increase in JNK activity, despite their diverse biological responses. Since IGF-I also enhances Akt activity in MCF-7 cells, stable transfections with JNK3 and Akt were performed to more clearly delineate the importance of JNK and Akt in IGF-I cellular survival. Overexpression of either Wt JNK or Mt Akt lead to increases in chemotherapy induced cell death and reduced growth factor induced cell survival, confirming that Akt activity confers IGF-I survival and that enhanced JNK3 signaling reduces IGF-I survival effects. PI 3-kinase is an upstream mediator of IGF-I stimulation of both Akt and JNK and thus may

indirectly influence the biological outcome of opposing stimuli and the strength of IGF-I effects. Overexpression of Wt JNK3 demonstrates that increased JNK signaling by Taxol or IGF-I treatment further enhances Akt response, placing JNK upstream of Akt. Our model reflects a realistic scenario where cells encounter environmental signals that convey opposing consequences. Our results support an important role for JNK in transducing apoptotic responses in tumor cells but also strongly suggests that PI 3-kinase signaling may further convey complex growth factor and stress responses via downstream interactions between JNK and Akt.

Materials and Methods

Cell Culture and Treatments-- MCF-7 cells were provided by C. Kent Osborne (San Antonio, TX) and were maintained in full media (IMEM without phenol red (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA), antibiotics, glutamine, and insulin). In each experiment cells were plated in full media and cultured overnight at 37°C and 5% CO_2. The following day cells were washed twice with warm PBS (Biofluids, Rockville, MD) and then cultured overnight in SFM. The next day cells were treated with IGF-I (Bioreclamation, Hicksville, NY), Taxol (paclitaxel (Mead Johnson, Princeton, NJ)), Taxotere (docetaxel (Rhone-Poulenc Rorer Pharmaceuticals, Collegeville, PA)) or doxorubicin (Gensia Laboratories, Ltd., Irvine, CA) as described in figure legends. Pretreatment with either 100 nM wortmannin or 50 μM LY294002 (Calbiochem and Alexis Biochemicals, respectively, San Diego, CA) was performed 40 minutes prior to stimulation with IGF-I. All protein concentrations from cell extracts were determined using a Bio-Rad D/C protein assay kit (Bio-Rad, Hercules, CA).

Transfection experiments: Hemagglutinin (HA) tagged Akt and Akt (K179A) vectors were graciously provided by M.E. Greenberg (9)). JNK3 and JNK3 (T183A/Y185F) pcDNA3 constructs were obtained from C. Franklin, University of Colorado Health Sciences Center. Generation of both wild-type (Wt) and mutant (Mt) Akt and JNK3 stable transfectants was performed by plating 2 x 10^6 of MCF-7 cells per 10 cm dish. The following day, cells were transfected with 10 μg of DNA by lipofection (Lipofectamine, Gibco BRL, Grand Island, NY). Control cells were transfected with empty vector. Twenty-four to 48 hours later cells were selected with 800 μg/ml of G418. Individual resistant colonies were isolated and expanded. Detection of clones overexpressing either Akt and JNK3 Wt or Mt genes was performed by Western blot analysis using HA primary antibody (clone 3F10, Boehringer Mannheim, Indianapolis, IN).

JNK assay and Western blot analysis--As a positive control for JNK activation, cells were plated and serum starved as described previously. UV irradiation was performed by exposing cells to UVC (50 J/m^2) in a Strategene UV linker 1800 (Stratagene, La Jolla, CA). Cells were then incubated at 37^{0}C in 5% CO_2 for 50 minutes before preparing cell lysates. Other cells were plated and serum-starved and then exposed to either IGF-I and/or chemotherapy as indicated. Tissue culture dishes were washed twice with ice-cold PBS. Cells were harvested and *in vitro* kinase assays were performed as described previously (15) using cell lysate volumes corresponding to 600 μg of total protein. The products were then resolved by 10% SDS-PAGE.

The gel was dried and subjected to radiography. Additionally, phosphorylated c-jun product incorporating ^{32}P was quantitated by PhosphorImager analysis.

To assure that kinase reactions in each experiment contained approximately equal amounts of JNK protein per sample, 10 µl of supernatant from each kinase reaction were run on a 10% SDS-PAGE. Proteins were transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) and JNK protein quantity was verified by immunoblotting with JNK1 antibody (FL, Santa Cruz Biotechnology, Santa Cruz, CA) and enhanced chemiluminescence (Amersham, Piscataway, NJ).

Akt substrate construct, affinity purification, immobilized and soluble GST thrombin-Akt substrate- The peptide RPRAATF, corresponding to the sequence containing the threonine phosphorylation site of GSK-3, was fused in frame with GST in pGEX-4T-1 by first hybridizing the oligonucleotides (5'-AATTGCGTCCGCGTGCTGCCACCTTCG-3') and (5'-AATTCGAAGGTGGCAGCACGCGGACGC-3') to produce a double stranded DNA with EcoRI sticky ends. This DNA fragment was then cloned into the EcoRI site of pGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ), regenerating only one EcoRI site. A construct with the insert in the correct orientation was identified by restriction analysis and sequencing (University of Colorado Cancer Center DNA Sequencing Core Service). Affinity purification of the GST thrombin-Akt substrate fusion protein was performed as described by Smith et al. (16).

Akt assay- After IGF-I treatment, tissue culture dishes were washed twice with ice-cold PBS. Cells were harvested in lysis buffer and kinase assays performed as previously described (17) using volumes of pre-cleared lysate containing 400 µg of total protein for each sample.

Reactions were terminated with 15 µl of 4X sample buffer. The products were then resolved by 10% SDS-PAGE. The gel was dried and subjected to radiography. Additionally, phosphorylated product incorporating ^{32}P was quantitated by PhosphorImager analysis. Immunoprecipitation or Western blot analysis of Akt1 was performed using anti-Akt1 antibody (C20, Santa Cruz Biotechnology, Santa Cruz, CA).

Apoptosis Assays: 75,000 MCF-7 cells were plated in each 1.7 cm^2 chamber of Biocoat CultureSlides™ (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). Cells were cultured overnight as described above. The following day, cells were washed twice with PBS and then exposed to SFM control, IGF-I alone, Taxol alone, or Taxol plus IGF-I as described in figure legends. Forty-eight hours after exposure to treatment cells were fixed in 3% formaldehyde in PBS for five minutes, washed three times with PBS, and nuclei were stained with a solution containing 15 µM Hoechst 33258 in PBS for 10 minutes. A minimum of 200 cells were counted per treatment and the nuclei morphology determined as viable or apoptotic using a fluorescence microscope (Zeiss, Oberkochen, Germany).

Data Analysis: Apoptosis assays were analyzed by multiple logistic regression performed using SAS/PROC GENMOD, version 6.12 (SAS Institute, Carey, NC). Tests were performed for overall effect of IGF or the transfected gene on Taxol induced apoptosis, as well as for significant interaction effects between IGF and the transfected gene. As multiple comparisons were required to address all questions of interest, results were not considered statistically significant unless the contrast reached $p < 0.005$.

Results

The various biological outcomes of IGF-I treatment of breast cancer cells have been well described. However, less is known about the signaling pathways that allow IGF-I to convey its cytoprotective effects in chemotherapy treated cancer cells. We initially set out to measure JNK response to chemotherapy in our model and then to determine if IGF-I treatment might suppress stress-related signaling as a possible mechanism for its inhibition of programmed cell death. JNK response to chemotherapy treatment has also been suggested to correspond to cell sensitivity to chemotherapy (18). Therefore, we tested whether Taxol, Taxotere or doxorubicin exposure induced JNK activity in MCF-7 cells. Cells were harvested at various time points to determine the peak time of activation. Figure 1A illustrates that doxorubicin treatment of cells increases JNK activity typically less than 3-fold. Use of lower concentrations of doxorubicin showed a similar time and level of activation as that illustrated in Figure 1 with doxorubicin 0.8 μM, suggesting that JNK response does not directly mediate doxorubicin cytotoxicity. Taxol and Taxotere treatment of cells induced JNK activity to a greater extent than doxorubicin. A bimodal pattern of JNK activation was observed with Taxol treatment with early activation occurring at two hours of exposure and maximal activation observed at eight hours. Taxotere treatment led to similar levels of JNK induction as Taxol (≥ 3-fold over control), however only one peak activation time occurred at two hours of exposure.

In order to study IGF-I's ability to regulate JNK in breast cancer cells, the effect of IGF-I treatment alone on JNK activity was measured. Initially, we predicted that IGF-I treatment alone would either, 1) suppress JNK signaling, since many treatments that stimulate JNK result in cell

death and IGF-I opposes this response, or 2) have minimal effect on JNK, as other investigators have shown that tyrosine kinase receptor activation leads to low levels of JNK activity, generally peaking within 30 minutes of exposure (19-23). Figure 2 shows that IGF-I potently stimulates JNK. Maximal JNK activity was observed after one to two hours of exposure, when 7- to 9-fold levels of induction were typically observed in multiple experiments.

We then assessed JNK response to co-treatment using IGF-I and chemotherapy to determine if these two treatments might counteract one another's signaling through JNK. UV treatment was also studied in co-treatment of cells to ascertain if IGF-I effects on JNK agonists could be extended to other stress treatments. Cells were pretreated with either Taxol (for five or seven hours) or Taxotere (for 1 hour) then IGF-I was added for the last hour of exposure, in order to harvest cells at the approximate times of maximal activity for each treatment. Cells were also treated by adding both IGF-I and chemotherapy simultaneously, as performed in previous cell viability and apoptosis assays (24) and herein, and harvested at peak times for each stress treatment: Taxol, Taxotere or UV. Wortmannin was used to determine if IGF-I activation of JNK was reversed by PI 3-kinase inhibition. Figure 3A, B, and C show JNK phosphorylation of c-Jun substrate under the conditions described above. In contrast to a previous report (12) and our predictions, IGF-I co-treatment of cells did not interfere with JNK activation by stress treatments. In fact, JNK induction was higher with co-treatment, when IGF-I was added after stress treatment or simultaneously, than with any treatment alone. Addition of wortmannin to IGF-I containing samples significantly reduced IGF-I dependent activation of JNK even in cells co-treated with Taxol, supporting the hypothesis that PI 3-kinase lies upstream of JNK with IGF-

I treatment. The greatest JNK activity was observed when cells were treated for only the duration of time seen for maximal activity with each treatment alone. IGF-I induction of JNK over that of Taxol alone was minimal with more prolonged exposure when treatments were added simultaneously (Figure 3A). Finally, Figure 3B confirms that co-treatment of IGF-I with Taxotere or UV also results in enhanced JNK activity beyond either treatment alone.

We then assessed the response of both PI 3-kinase and Akt to IGF-I treatment for two reasons. First, since IGF-I treatment effects on JNK were, in part, reversed by PI 3-kinase inhibition, we wanted to test if IGF-I induced PI 3-kinase in our model. Second, IGF-I stimulation of both PI 3-kinase and Akt kinase may also be important since several other investigators have reported that IGF-I induces its survival effect via these kinases (25). MCF-7 cells were treated with IGF-I for 10 minutes and analyzed using a PI 3-kinase *in vitro* assay. IGF-I treatment resulted in a large increase in the phosphorylated substrate, phosphatidylinositol-3P. Pretreatment of cells with wortmannin prior to IGF-I treatment inhibited induction of PI 3-kinase (data not shown).

To further characterize the role of PI 3-kinase action in MCF-7 cell survival, we developed a kinase specific substrate to investigate the involvement of downstream Akt activity in PI 3-kinase survival properties. *In vitro* kinase assays in Figure 4 show that IGF-I treatment of MCF-7 cells induces Akt maximally at 10 minutes of exposure and that pretreatment with PI 3-kinase inhibitors, LY294002, or wortmannin (data not shown), reduced IGF-I induction of Akt (Figure 4A and B). Increased Akt activity occurred both when Akt was isolated by immunoprecipitation (data not shown), and when Akt containing lysates were exposed to an immobilized Akt specific

substrate, GST-GSK, following IGF-I treatment (Figure 5). These results further support that PI 3-kinase and Akt may elicit IGF-I survival effects in MCF-7 cells.

In order to more clearly decipher the potential role of either JNK or Akt in IGF-I cytoprotection, MCF-7 cells were stably transfected with either Wt Akt, Wt JNK3, Mt Akt (K179A), or Mt JNK3 (T183A/Y185F) hemagglutinin tagged constructs. Cells overexpressing transfected protein were plated in chamber slides, serum starved overnight and treated cells were exposed to Taxol or IGF-I treatment alone or in combination. Figure 5A shows that IGF-I significantly reduced apoptosis induced by Taxol, (test for overall effect, $p<0.0001$). IGF-I reduced cell death in parental and Mt Akt transfected cells by 59% and 53%, respectively, compared to Taxol treatment alone. Cells overexpressing Wt Akt experienced only a non-significant 34% reduction in apoptosis with IGF-I treatment, ($p=0.056$). The lack of a significant effect of IGF-I on Wt Akt expressing cells appears to be due largely to the dramatic effect that overexpression of Wt Akt has on suppressing the ability of Taxol to induce apoptosis in these cells. Wt Akt overexpressing cells underwent 70% less apoptosis than parental MCF-7s treated with Taxol (13% vs 44%, $p < 0.0001$). There was no significant difference between Mt Akt transfectants and parental cells with respect to IGF-I protection from Taxol induced apoptosis. These latter results suggest either that another pathway may convey IGF-I survival properties or that Mt Akt overexpression was not able to squelch endogenous Akt signaling in response to IGF-I.

Cells transfected with Wt or Mt JNK3 were treated in a similar fashion as described above but were performed in separate experiments. Overall, parental control treatment groups were very

similar to those in Akt experiments; in JNK experiments IGF-I reduced Taxol induced apoptosis by 60% versus 59% in Akt experiments, Figure 5A and B. Comparison of all treatment groups of Wt JNK3 transfectants to parental cells demonstrates that overexpression of Wt JNK3 results in a significant increase in apoptosis (test for overall effect, p=0.0001).

Figure 5B also shows that, similar to the effects of Wt Akt, transfection of cells with Mt JNK3 leads to a dramatic decrease in cell sensitivity to Taxol compared to parental cells, 19% versus 47% apoptosis (p=0.0009). This lends further support to the hypothesis that JNK activity is transducing a cell death signal under cellular stress. Similar to its effects on Wt Akt transfected cells, IGF-I did not significantly inhibit Taxol induced cell death in Mt JNK3 transfectants.

Given that IGF-I induction of JNK was inhibited with wortmannin and that increased expression of Wt JNK3 did not enhance IGF-I protective properties, we set out to determine if one kinase might function upstream of the other in response to IGF-I. In order to test if Akt functions upstream of JNK in IGF-I signaling, parental and Wt Akt transfectants were exposed to IGF-I for two hours and JNK activity was measured using *in vitro* kinase assays. JNK activity in untreated or IGF-I treated cells was not significantly altered in Akt overexpressing cells compared to parental cells (data not shown), showing that Akt does not convey IGF-I mediated signaling to JNK. We also tested if JNK3 may function upstream of Akt to alter Akt response. Akt activity was measured and compared in parental MCF-7 cells and Wt JNK3 transfectants to determine a). if Taxol treatment alone affects Akt activity, b). if Taxol treatment alters Akt response to IGF-I, and c). if overexpression of Wt JNK3 mediates Akt response to Taxol or IGF-I. Figure 6

illustrates that IGF-I treatment for 10 minutes induces Akt in parental MCF-7 cells, as shown previously (Figure 6A, Time 0 of Taxol exposure). Further comparison of Akt activity of control (serum starved) parental MCF-7 cells to Wt JNK3 transfectants shows that overexpression of JNK3 enhances ligand-independent Akt activity. IGF-I treatment of JNK3 transfectants leads to a lower fold induction of Akt activity compared to parental controls. Treatment with Taxol alone resulted in enhanced Akt activity in both parental MCF-7 and JNK3 transfectants. Further enhancement of Akt activity was typically observed when cells were exposed Taxol and then treated with IGF-I for 10 minutes prior to harvesting. In a separate experiment, cells were exposed Taxol 0.25 μM in a time dependent fashion. Western blot analysis of Akt protein levels in MCF-7 cells shows that Taxol exposure does not alter Akt protein levels. Thus, increases in Akt activity from Taxol exposure do not result from increased Akt expression.

Discussion

In this paper we demonstrate that IGF-I has potent effects on JNK and Akt activity using *in vitro* kinase assays. IGF-I activation of both PI 3-kinase and Akt supports the hypothesis that this pathway is an important pathway for IGF-I mediated chemoprotection. These data along with apoptosis experiments using Wt and Mt Akt transfectants further confirm our previous results showing that pharmacologic inhibition of PI 3-kinase and its downstream kinases enhanced both doxorubicin and Taxol induced apoptosis (24). The significant amount of protection provided by Wt Akt overexpression alone, in the absence of IGF-I, we believe results from constitutive activity of the overexpressed protein, thus reducing Akt requirement for ligand in these cells. We observe a low level of Akt activity in our parental MCF-7 cells. These results suggest that

Akt constitutive activity may play some role in breast cancer cell survival but that the presence of growth factor may, in some situations, further augment Akt response. Interesting, however, was our observation that Wt JNK transfectants also enhanced Akt activity in the absence of any treatment but these transfectants experienced slightly higher rates of apoptosis, suggesting again that the opposing functions Akt and JNK may be closely linked but exactly how JNK regulates the biological outcome of Akt remains to be described.

Ultimately Akt may convey its survival effects by inhibition of the activity of the pro-apoptotic protein BAD (9). Phosphorylation of BAD by Akt results in increased BAD dimerization to the protein 14-3-3 and decreased BAD dimerization to either Bcl-2 and Bcl-X_L. The ultimate outcome is reduced pro-apoptotic activity of BAD (26, 27). Modulation of Bcl-2 related proteins may also have important consequences to cell chemosensitivity via JNK signaling. For example, others have shown that JNK may phosphorylate Bcl-2 (28) or that Bcl-X_L may block JNK activation in some models (29). To add further to the complexity of this model, both Bcl-X_L and Akt may function to prevent caspase 9 cleavage to its pro-apoptotic form (10, 30, 31). Therefore, Akt response in the presence of various forms of cellular stimuli may be further mediated through crosstalk of signaling pathways or through protein complex formation with constituents of other signaling pathways involving kinases like JNK.

Activated PI 3-kinase has been previously shown to convey messages through JNK after exposure to growth factors like PDGF, and in some cases EGF, but not after exposure to UV irradiation or osmotic shock (32, 33). Since IGF-I survival effects are primarily PI 3-kinase

dependent (25) and since it activates JNK in MCF-7 cells, it is then plausible that JNK may convey some IGF-I cytoprotection in a PI 3-kinase dependent fashion. However, results from our present apoptosis experiments do not support this hypothesis. IGF-I actually lost its significant cytoprotective effect in cells overexpressing JNK3, where the stochiometry of JNK3 versus Akt protein levels may have allowed JNK3 induced apoptotic response to overcome some of IGF-I effects in these cells. Surprisingly, JNK3 overexpression and Taxol treatment resulted in increased Akt activity, suggesting either that Akt activity could not overcome the apoptotic effects of JNK and Taxol or that Akt has some role in cell death.

Considering the dramatic increase in JNK activity induced by IGF-I, there is still great interest in determining if JNK mediates other IGF-I induced responses. Similar to PDGF (33, 34), Rac is a likely intermediate of IGF-I induced PI 3-kinase and JNK activation. The large degree of JNK activation by IGF-I was somewhat surprising since activation of other tyrosine kinase receptors does not typically induce the same level or time to maximal stimulation as we observed (19-23). For example, other investigators (35, 36) have reported that insulin treatment of CHO or Rat 1 HIR fibroblasts activates JNK by 10 to 15 minutes of exposure and this activity results in an increase in AP-1 DNA binding and may enhance cell proliferation (35). Given that IGF-I induction of JNK is notably higher than what has been reported with other growth factors and since other growth factors induce proliferation through JNK (37), we are in the process of determining if our JNK3 transfectants demonstrate a greater proliferative response to IGF-I. Thus far, it does not appear that this is the case (unpublished results).

In comparing the level of JNK response induced by IGF-I in our model with that reported by other investigators, considerations of cell type and study design are important. IGF-I treatment appears to have minimal effects on JNK signaling in 293 and KB cells (38). Despite IGF-I's limited ability to induce JNK in embryonic kidney 293 cells, pre-treatment with IGF-I suppresses JNK stimulation by TNF-α and anisomycin (12). These investigators stressed the necessity of IGF-I pre-treatment in order to observe inhibition of JNK response to cellular stress. We did not study the effects of IGF-I pre-treatment in our model. Also, these investigators did not study JNK response to IGF-I treatment alone beyond one hour of exposure. We observed maximal activity after one to two hours of IGF-I exposure. Given these mixed results reported by others, we initially anticipated that IGF-I might abrogate chemotherapy induction of JNK, if it had any measurable effect on JNK. It is now clear that although chemotherapy drugs activate JNK, IGF-I induced JNK to a far greater extent in our model. Co-treatment with chemotherapy and IGF-I further enhanced JNK activity, even though they have opposing effects on breast cancer cell survival. The biological effect of increased JNK signaling through overexpression of JNK3 in our model suggests that even though it was induced both by Taxol and IGF-I, JNK3's primary effect in either case appears to be pro-apoptotic rather than protective – this is shown by the reduced anti-apoptotic effect caused by IGF-I in the Taxol treated Wt JNK3 transfectants, compared to the parental cells (see Figure 5B).

However, there may be additional reasons for our inability to detect a survival effect of IGF-I through JNK. First, JNK3 may not be the specific JNK protein responsible for the IGF-I induction of JNK activity that we observed. JNK1 and JNK2 may have been better candidates

but thus far the literature does not support the function of one protein over any other for conveying growth factor responses. Another reason could be that different JNK agonists result in phosphorylation of specific substrates that may ultimately dictate biological responses through JNK (39, 40). These experiments were not designed to address if IGF-I treatment may result in phosphorylation of different transcription factors and altered gene expression compared to stress treatment. Finally, JNK and Akt function or activity may be somewhat dependent upon one another. Obviously, JNK signaling is a complex model where regulation of response may occur at many levels. However, we feel that the most likely hypothesis is that IGF-I mediated activation of JNK is not the main determinant of IGF-I's anti-apoptotic properties, but it may be an intermediate of another cellular response involving Akt. It seems likely that IGF-I anti-apoptotic effects are mediated by the constituents of protein complexes formed after exposure to specific external stimuli. Our data support that JNK is likely to regulate Akt response by functioning upstream of Akt in a PI 3-kinase sensitive fashion.

We did not observe a large induction of JNK with doxorubicin treatment, as reported by others (41). These differences in response to either IGF-I or doxorubicin in our model may possibly be explained either by differences in cell sensitivity to doxorubicin or the absence of MDR overexpression in our MCF-7 breast cancer cells. Furthermore, DNA damage by chemotherapy drugs may not be required in order for chemotherapy drugs to induce JNK, for example, the microtubulin interfering agents like Taxol, Taxotere, vincristine, and others also stimulate JNK (13) via upstream Ras and ASK1 proteins (13). JNK mediates cell death in response to various environmental stresses and may phosphorylate and stabilize the p53 tumor suppressor protein

when activated by DNA damage (42, 43). Thus, p53 status of cells may further impact the biological response to JNK activation by chemotherapy drugs or IGF-I cytoprotection through caspase 9, a downstream target of p53 (44). Results from other investigators suggest that JNK responses and chemotherapy induced apoptosis are further augmented by oncogenic transformation of cells (45). Therefore, JNK is an important target to further characterize as a mediator of chemosensitivity.

Finally, given that both stress and IGF-I treatment induced JNK then regulation of the diverse biological outcomes of these treatments may also occur by the differences in the strength or duration of JNK signal, like that previously described with MAPKs (46). However, we do not believe this to necessarily be the case, since the strength of IGF-I activation of JNK was intermediate between the apoptotic stimuli of UV and chemotherapy and since the time to maximal activity for IGF-I treatment was delayed for one to two hours like UV and Taxotere treatment.

Clearly, the biological role(s) of JNK are controversial (see review by Minden and Karin (47)). Simple activation of JNK by any agonist does not imply the same biological response. JNK activity may either convey resistance or sensitivity to cellular stress (12, 48) or even proliferative responses (37). Some investigators have begun to study the effects of diverse stimuli on kinases upstream of JNK and differential regulation resulting from the various JNK isoforms derived from the three JNK genes, JNK1, JNK2 and JNK3 (38, 40) to understand the various biological responses associated with JNK activity. Our findings that JNK may lie upstream of Akt may

significantly affect the biological outcome of JNK activation. We also plan to address the influence of different JNK isoforms in our model.

In conclusion, we propose that IGF-I induction of PI 3-kinase is influencing pathways other than Akt to directly alter cellular stress responses. Clearly, regulation of cell death signals by growth factors permit tumor cells to endure environmental stresses, whereas blocking growth factor action may enhance apoptotic death. We show here that enhancing the activity of IGF-IR dependent pathways like Akt lessens chemosensitivity of breast cancer cells. We believe this interaction is not determined simply by activation of Akt but may also be regulated through JNK (and ultimately Akt) or by JNK phosphorylation of its various substrates, depending upon cellular environment and the presence of disparate stimuli. Further study is needed to ascertain these complex signaling networks and what conditions determine cellular outcome to ultimately enhance the efficacy of chemotherapy drugs in killing tumor cells.

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Footnotes

* This work was supported in part by Susan G. Komen Foundation (to C.L.V.D.B.); the Burroughs Wellcome Fund/American Foundation for Pharmaceutical Education (to C.L.V.D.B.); and American College of Clinical Pharmacy/Rhone-Poulenc Rorer (to C.L.V.D.B.); and United States Army Medical Research and Command Grant DAMD17-99-1-9142 (to C.L.V.D.B). The content of this information does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

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[1] The abbreviations used are: IGF-IR, insulin-like growth factor receptor; IRS, Insulin Receptor Substrate; MKK, MAP kinase kinases; MAPK, mitogen activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase, JNK (or SAPK), c-Jun N-terminal kinase (or stress-activated protein kinase); IMEM, Improved Minimal Essential Medium; SFM, serum free media; PIPES, piperizine-*N,N'*-bis-(2-ethanesulfonic acid); PNPP, p-nitrophenyl phosphate; PBS, phosphate buffered saline; GSK, glycogen synthase kinase-3; GST, glutathione S-transferase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography; UV-C, ultraviolet-C; PDGF, platelet-derived growth factor; BAD, BCL-X_L/BCL-2 associated death promoter; ASK1, apoptosis signal-regulating kinase.

Figure Legends

Figure 1. Taxol and Taxotere treatment increases JNK activity. Cells were treated with either doxorubicin (not shown graphically), Taxol (■), or Taxotere (▲) and harvested in a time-dependent fashion. UV was used as a positive control for JNK activation in MCF-7 cells. Analyses of phosphorylated c-$Jun_{(1-79)}$ resulting from *in vitro* kinase assays are shown. The graph shows the combined results of two independent experiments (points: mean; bars: range).

Figure 2. JNK activity is dramatically increased with IGF-I treatment. MCF-7 cells were treated with IGF-I in a time-course experiment. Analyses of phosphorylated c-$Jun_{(1-79)}$ resulting from *in vitro* kinase assays are shown. UV was used as a positive control for JNK activation in MCF-7 cells. The graph shows the combined results of two independent experiments (mean ± range).

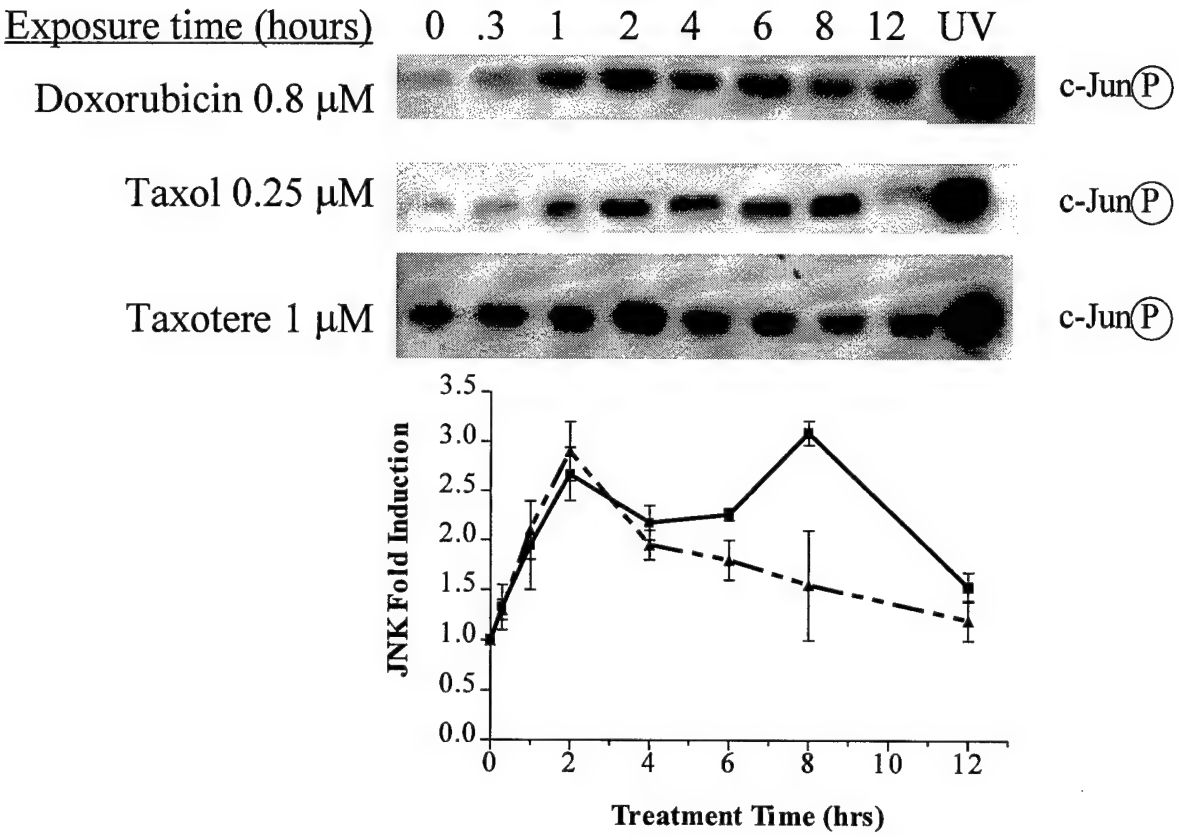
Figure 3. Co-treatment IGF-I and chemotherapy results in additive effects on JNK. A. Cells were exposed to each treatment until time of maximal response for each treatment. Also, JNK activity was measured by exposing cells to Taxol and IGF-I simultaneously. Some IGF-I co-treated cells were exposed to wortmannin prior to IGF-I. Western blot analyses of kinase reactions were performed to compare JNK protein expression in each treatment. **B.** The effect of IGF-I co-treatment with Taxotere and UV on JNK activity was tested by exposing cells to each treatment until time of maximal response of each treatment. Western blot analysis was performed to compare JNK expression in each treatment. Each graph shown represents three independent experiments (mean ± s.e.m.).

Figure 4. GST-Akt peptide substrate phosphorylation. GST-Akt specific substrate (GST-

GSK) was developed as described in Methods. *A.* Cells were treated with IGF-I and at the indicated times as described in Methods. Phosphorylated GST-GSK product resulting from *in vitro* kinase assays were detected and measured. The graph shown represents three independent experiments (mean ± s.e.m.) *B.* MCF-7 cells were either pre-incubated in 50μM LY294002 for 40 minutes prior to IGF-I treatment or treated with IGF-I alone, as indicated, for three (Lanes 2 and 3) to five minutes (Lanes 4 and 5). Phosphorylated GST-GSK was analyzed using SDS-PAGE and autoradiography.

Figure 5. Overexpression of Mt Akt or Wt JNK3 enhanced Taxol induced apoptosis and reduced IGF-I survival effects. *A.* Parental cells and Wt or Mt Akt transfectants were plated in chamber slides and treated as described in Methods. Forty eight hours after exposure cells were stained with Hoechst 33258 for analysis of apoptotic nuclei. *B.* Parental cells and Wt or Mt JNK3 transfectants were tested in the same fashion as Akt transfectants above. Bars are the average of three independent experiments for both Akt and JNK experiments. Error bars show 95% C.I. around the mean.

Figure 6. Taxol and JNK overexpression lead to increased Akt activity. *A.* Parental MCF-7 or Wt JNK transfectant cells were treated with Taxol for indicated times and then treated with or without IGF-I for 10 minutes. Cells were harvested as described in Methods for Akt kinase assays. Phosphorylated GST-GSK product resulting from *in vitro* kinase assays were detected and measured. The graph illustrates one representative experiment. B. MCF-7 cells were treated with 0.25 μM Taxol for indicated times. 40 μg of total cell lysate were separated on 10% SDS-PAGE gel and probed with an anti-Akt1 antibody.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c-Jun(P)
Taxol 0.25 μM
c-Jun(P)
Taxotere 1 μM
c-Jun(P)
JNK Fold Induction
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3.0
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2.0
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1.0
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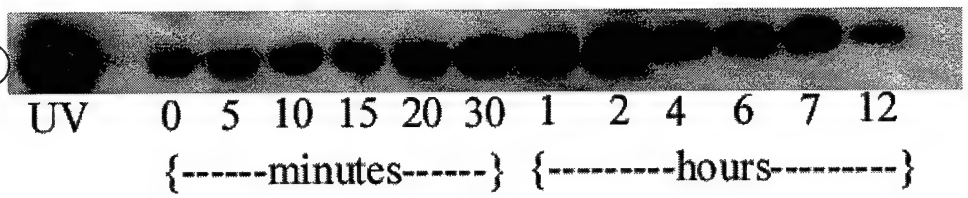
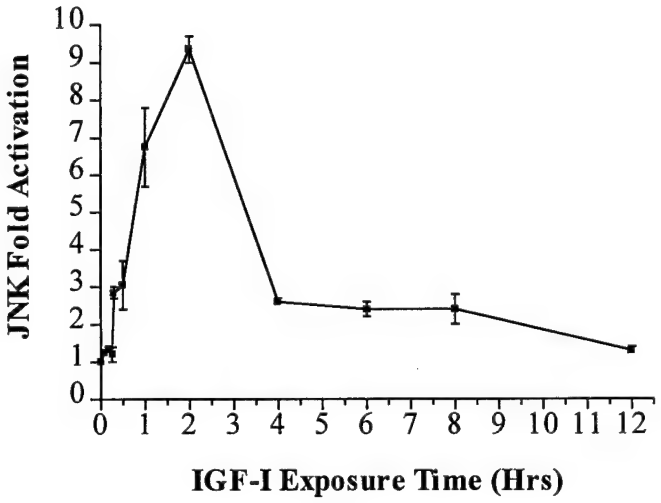
0 2 4 6 8 10 12
Treatment Time (hrs)

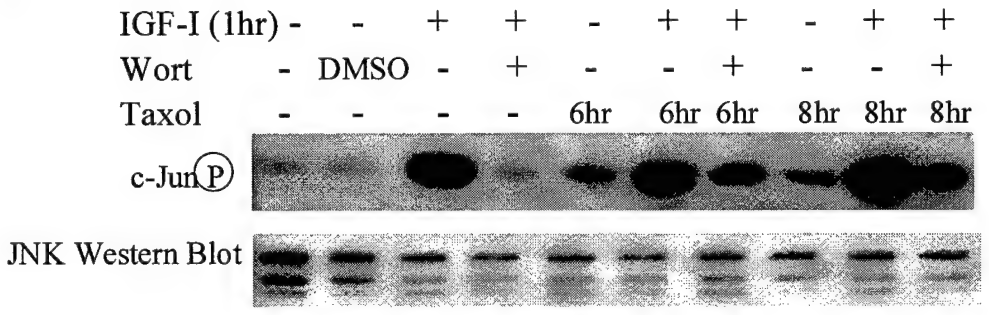
A.

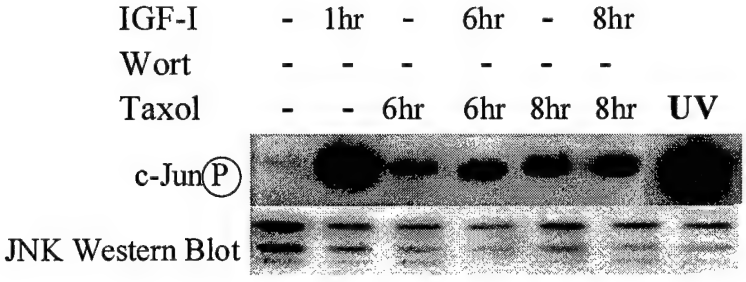


IGF-I Exposure Time



A.


IGF-I (1hr) - - + + - + + - + +
Wort - DMSO - + - - + - - +
Taxol - - - - 6hr 6hr 6hr 8hr 8hr 8hr
c-Jun(P)
JNK Western Blot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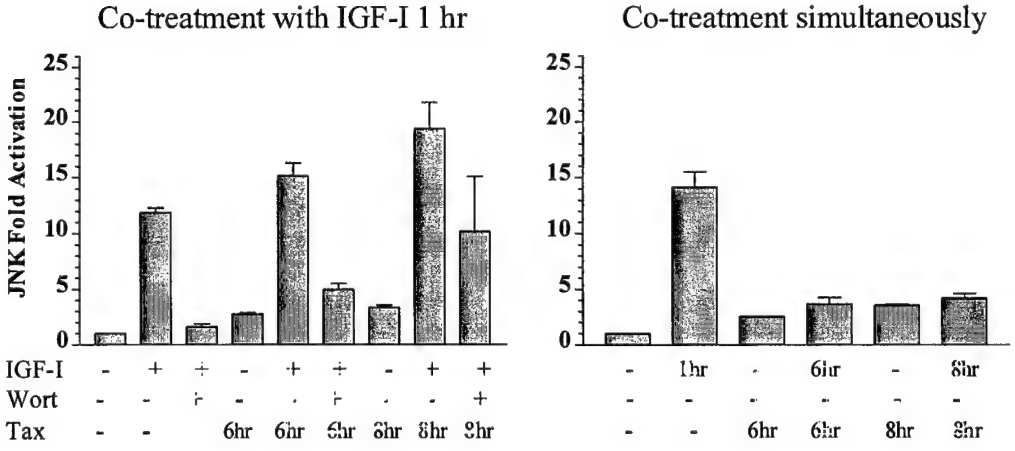
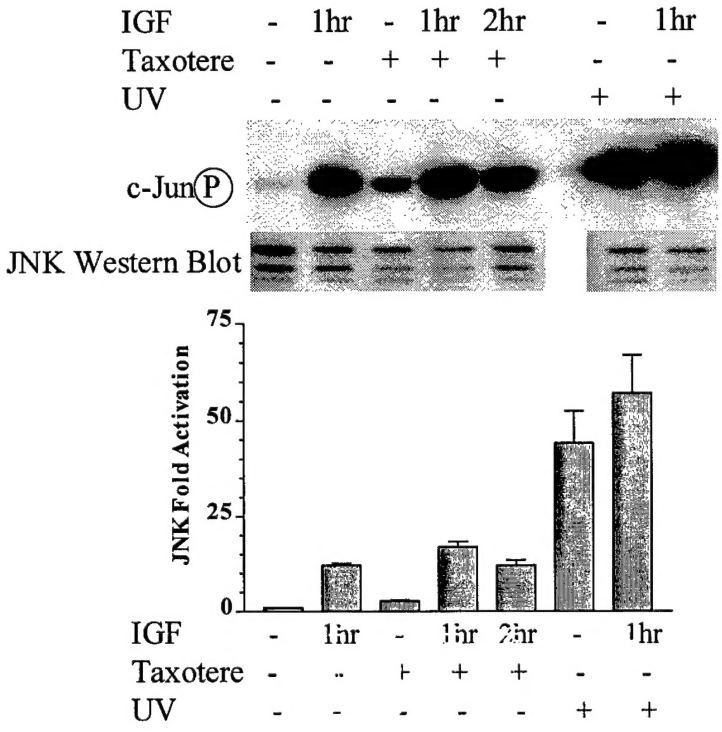
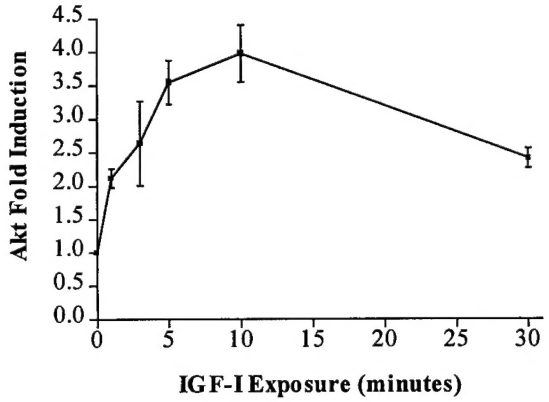
JNK Western Blot


Co-treatment with IGF-I 1 hr
Co-treatment simultaneously
JNK Fold Activation
25
20
15
10
5
0
IGF-I - + + - + + - + +
Wort - - + - - + - - +
Tax - - 6hr 6hr 6hr 6hr 8hr 8hr
- 1hr - 6hr - 8hr
- - - - - -
- - 6hr 6hr 8hr 8hr

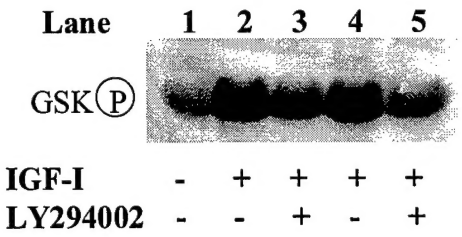
B.


IGF - 1hr - 1hr 2hr - 1hr
Taxotere - - + + + - -
UV - - - - - + +
c-Jun(P)
JNK Western Blot
JNK Fold Activation
75
50
25
0
IGF - 1hr - 1hr 2hr - 1hr
Taxotere - - + + + - -
UV - - - - - + +

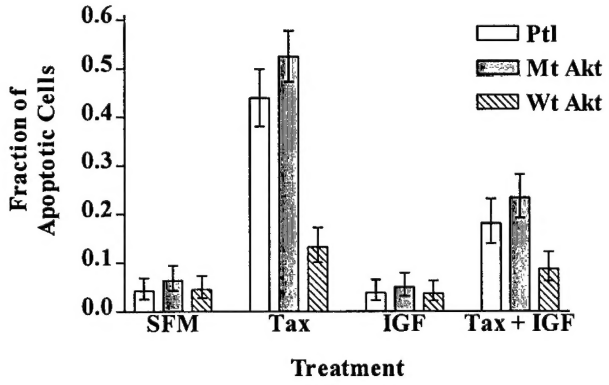
A.


Akt Fold Induction
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
IGF-I Exposure (minutes)

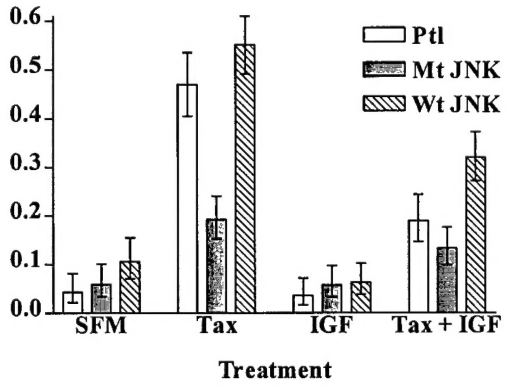
B.


Lane 1 2 3 4 5
GSK (P)
IGF-I - + + + +
LY294002 - - + - +

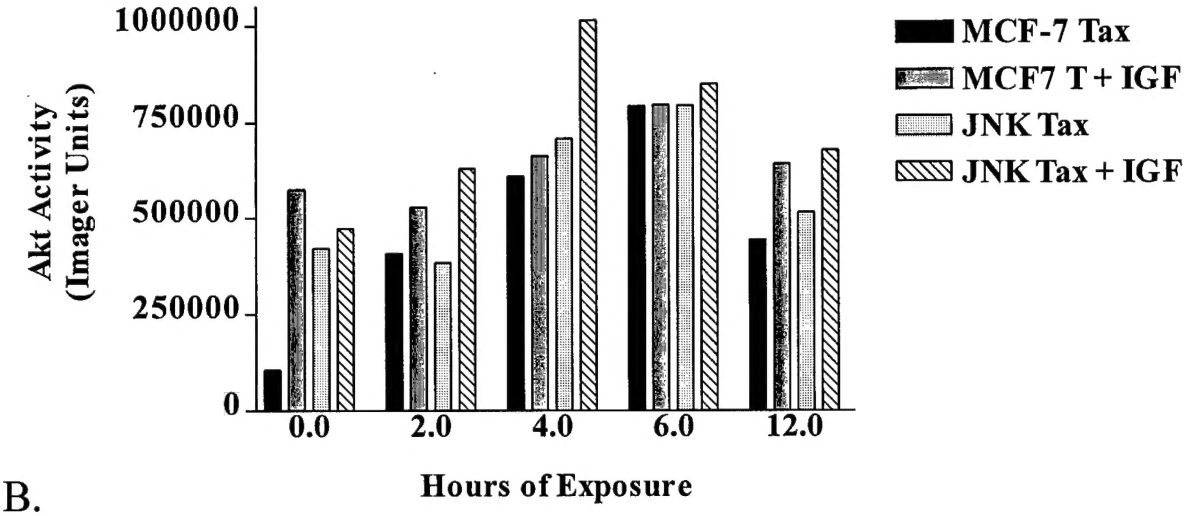
A.


Fraction of Apoptotic Cells
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ptl
Mt Akt
Wt Akt
SFM
Tax
IGF
Tax + IGF
Treatment

B.


0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ptl
Mt JNK
Wt JNK
SFM
Tax
IGF
Tax + IGF
Treatment

A.


1000000
750000
500000
250000
0
Akt Activity (Imager Units)
0.0
2.0
4.0
6.0
12.0
Hours of Exposure
MCF-7 Tax
MCF7 T + IGF
JNK Tax
JNK Tax + IGF

B.


Taxol exposure time (hrs) 0 2 4 8 16 24